REMARKS

Applicant has canceled claims 23, 50 and 51 without prejudice and expressly reserve the right to pursue the subject matter of the canceled claims in one or more subsequent applications.

Applicant has amended claims 15, 17, 19 and 38 to recite "...intramyocardially delivering bone marrow stromal cells modified ex vivo to produce an angiogenic factor...." Support for these amendments is found in the specification on, e.g., page 9, lines 17-25.

Claims 15, 17, 19, 23-25, 38, 40, 50-51 and 54 stand rejected under 37 C.F.R. 112, first paragraph. In particular, the Examiner contends "the specification is enabling for intramyocardial delivery of autologous mesenchymal stem cells modified ex vivo to express an angiogenic factor via a constitutive promoter operatively linked to a sequence encoding an angiogenic factor to normal tissue adjacent to ischemic tissue in the myocardium" but contends that such "does not enable the administration of any cell type or delivery of cells to tissues not adjacent to the ischemic tissue or the absence of a transgene, any non-constitutive promoter or the use of any transgene in any cell type, for reasons of record." (Office Action, sentence spanning page 1-2) In view of the amendments to the claims and the following remarks, Applicant respectfully requests that the Examiner reconsider and withdraw the rejection of the claims.

> "A decision on the issue of enablement required determination of whether a person skilled in the pertinent art, using the knowledge available to such a person and the disclosure in the patent document, could make and use the invention without undue experimentation. It is not fatal if some experimentation is needed, for the patent document is not intended to be a production specification."
>
> *Northern Telecom, In. v. Datapoint Corp.*,15 USPQ 1321, 1229

Applicant has discovered that delivering an angiogenic factor to normal tissue adjacent to ischemic tissue in a diseased or ischemic heart stimulates collateral blood vessel formation and induces angiogenesis in the ischemic tissue and improves contractile function to a higher degree than is achieved by delivering an angiogenic factor directly to the ischemic tissue of the diseased or ischemic heart. Applicant's Examples 1-2 demonstrate that the improvements are achieved within 4 weeks after delivery of the angiogenic factor.

Applicant has taught that the foregoing effects, which were exemplified using an adenovirus construct encoding an angiogenic factor, can be achieved by delivering cells producing an angiogenic factor to normal tissue adjacent to the ischemic tissue in the heart (see, e.g. page 1, lines 8-10, page 5, lines 24-25 and page 6, lines 7-10). Applicant has taught particular cells capable of producing an angiogenic factor that are useful in this invention, and that such cells include bone marrow stromal cells (page 9, lines 17-19). Furthermore, applicant has taught that the cells can be manipulated, expanded or genetically engineered *ex vivo* to produce an angiogenic factor (page 9, lines 21-23). At the time of filing those of skill in the art could easily expand a population of bone marrow stromal cells using methods well known in the art and could easily genetically engineer bone marrow stromal cells to produce an angiogenic factor using methods well known in the art. In addition, those of skill in the art would expect that cells can be delivered directly to the heart target tissue by any well known method in the art. For example, Applicants have disclosed that the cells may be delivered e.g., via catheters (see e.g. page 12, lines 18 to 28) and Prittenger US Patent 6,387,369, column 4, lines 49-64 and col. 5, lines 38-39 e.g., demonstrate the direct injection of mesenchymal stem cells into the heart. Based on the successful results that Applicant achieved by delivering an angiogenic factor via an adenoviral vector to normal tissue adjacent to ischemic tissue and Applicant's teachings as they relates to the administration of cells producing an angiogenic factor, one of skill in the relevant art at the time this application was filed would have predicted that bone marrow stromal cells producing an angiogenic factor delivered intramyocardially to normal tissue adjacent to ischemic tissue in a diseased or ischemic heart would also achieve such results.

The Examiner contends that applicant's method requires that the cells engraft to achieve their desired effect. The Examiner further contends that only mesenchymal stem cells have been shown to engraft in the myocardium. Although Applicant respectfully disagrees with the Examiner's conclusions, Applicant, in the interest of expediting prosecution, has amended the cells to recite bone marrow stromal cells modified *ex vivo* to produce an angiogenic factor.

The Examiner also contends that the claims are enabled only for autologous cells. The Examiner is of the opinion that "immune responses to non-autologous cells would be expected, even if they did engraft" (Office Action 6/13/2006, page 4, second full paragraph) and "the Artisan would further conclude that such immune responses would be expected for any non-

autologous therapy as it is well known in the art that allogeneic and xenogenic cells also induce immune responses" (Office Action 9/21/2005). The Examiner's contentions are not well-founded. Applicants respectfully direct the Examiner to the multitude of persons who have been and are currently living with transplanted non-autologous organs, despite the potential for an adverse immune response to those organs. The existence of such survivors was know well before the filing date of this application. Thus, even if those of ordinary skill in the art speculated that an adverse immune response to non-autologous cells might occur, they would have known at the time of filing that methods routine in the art could counter such an immune response. Futhermore, one might have expected an adverse immune response to an adenoviral vector expressing an angiogenic protein and yet Applicant's Examples demonstrate that delivering an adenoviral vector expressing an angiogenic protein to normal tissue achieves the recited effects. Thus one of ordinary skill in the art in view of applicant's disclosure and the knowledge available in the art at the time of filing would have believed that non-autologous cells could also be used in the method as claimed.

Applicant also respectfully directs the Examiner's attention to Henning et al. "Human umbilical cord blood mononuclear cells for the treatment of acute myocardial infarction", Cell Transplan 2004; 13(7-8): 729-39)(enclosed). Henning et al. is a post filing reference, it states "the present experiments demonstrate that [human umbilical cord blood mononuclear progenitor cells] substantially reduce infarction size in rats without requirements for immunosuppression." Although Henning et al. is a post filing reference, it demonstrates that other cells in addition to mesenchymal stem cells, i.e., human umbilical cord blood cells (HUCBCs, not HUVECs as stated by Examiner), survive long enough in the myocardium to exert an effect on infarct size.

The Examiner identified a study by another , i.e., Lazarous et al. 1999, Cardiovasc. Res., 44:294-302 (Abstract), who injected adenovirus vectors into the pericardium and failed to see any improvement in myocardial collateral perfusion models. Based on the Examiner's analysis of that disclosure, the Examiner contends that applicant's claims are non-enabled unless the cells are delivered to normal tissue in the myocardium adjacent to ischemic tissue. The Examiner states, "Hence, the Artisan would not reasonably conclude that such factors would work, except when adjacent to the ischemic tissue."(Office Action 9/21/2005, page 11, paragraph 2) and "Therefore, the answer to the argument is that the cells must be delivered to sites immediately

adjacent, i.e., within the myocardium." (Office Action 6/13/2006, page 6, paragraph 1). Applicant believes that the Examiner has overlooked particular text in the pending claims and if such text is considered the Examiner will withdraw his comments.

Applicant's pending claims, in this response and in the previous response, clearly recite that the cells are delivered intramyocardially, thus "within the myocardium", and are delivered to normal tissue adjacent to the ischemic tissue. In view of the foregoing remarks, and Examiner's statements that "the Artisan would not reasonably conclude that such factors would work, except when delivered adjacent to the ischemic tissue", Applicant requests that the Examiner reconsider and withdraw this aspect of his rejection under 35 U.S.C. 112, first paragraph.

The Examiner had previously requested a further explanation of the dobutamine stress echocardiography recited in Example 2 (which is a well-established means for the diagnosis of myocardial abnormalities):

> Example 2 also argues that such administration provides for better wall motion; however, the results "indicate trends towards improvement in wall motion" (p. 15, paragraph 2). How this relates to the invention, and how these results demonstrate improved wall motion are unclear. The Examiner requests further explanation if Applicant wishes to use this information to support their claims.
>
> (Office Action, 9/11/2006, page 8-9).

Applicant provided an explanation of the echocardiography in the previous response, but the Examiner states "The Examiner thanks Applicant for the explanation, however [Pittenger and Martin, ("Mesenchymal Stem Cells and Their Potential as Cardiac Therapeutics," Circulation Research, (July 9, 2004) pp. 9-20) page 15-16] is not supplied so it is not considered." (Office Action page 6). Applicant regrets that the articles that were enclosed with the previous response were somehow lost in transit, such that the Examiner did not receive and therefore did not consider the references. For the Examiner's convenience, Applicant encloses another copy of Pittenger and Martin *supra* with this response. The dobutamine stress echocardiography disclosed in Example 2 is a well-established means for the diagnosis of myocardial abnormalities and is based on the visual detection of ischemia-induced radial wall motion abnormalities.

Those of skill in the art appreciate that improvements in wall motion are indicative of a less stiff more compliant ventrical and thus an improvement in heart function. Applicant directs the Examiner's attention to the review by Pittenger and Martin, *supra*, which also discuss improvements in wall motion as an indication of improvements in an infarcted heart. Claim 19 claims a method to increase contractile function of an ischemic heart. Improved wall motion correlates with increased contractile function in the heart. As such, one of ordinary skill in the art presented with data demonstrating increased wall motion would readily understand this to be a measure of increased contractile function.

In sum, Applicant has discovered that delivering an angiogenic factor to normal tissue adjacent to ischemic tissue in a diseased or ischemic heart stimulates collateral blood vessel formation and induces angiogenesis in the ischemic tissue and improves contractile function to a higher degree than is achieved by delivering an angiogenic factor directly to the ischemic tissue of the diseased or ischemic heart. Applicant has taught that these effects can be achieved by delivering cells producing an angiogenic factor to normal tissue in the ischemic or diseased heart (page 6, lines 7-10) adjacent to the ischemic tissue in the heart (see, e.g. page 1, lines 8-10 and page 5, lines 24-25). Applicant has disclosed particular cells that are capable of producing an angiogenic factor that are useful in this invention, and that such cells include bone marrow stromal cells (page 9, lines 17-19). Furthermore, applicant has taught that the cells can be manipulated, expanded or genetically engineered *ex vivo* to produce an angiogenic factor (see e.g., page 9, lines 21-23). Those of skill in the art at the time of filing could readily expand bone marrow stromal cells *ex vivo* and could also readily genetically engineer bone marrow stromal cells *ex vivo* if desired using methods routine in the art without resorting to undue experimentation. Applicants have disclosed that the cells may be delivered e.g., via catheters (see e.g. page 12, lines 18 to 28) and Prittenger US Patent 6,387,369, column 4, lines 49-64 and col. 5, lines 38-39 e.g., demonstrate the direct injection of mesenchymal stem cells into the heart.

Based on the successful results that Applicant achieved by delivering an angiogenic factor via an adenoviral vector to normal tissue adjacent to ischemic tissue, and on Applicant's teaching that cells producing an angiogenic factor delivered intramyocardially to normal tissue adjacent to ischemic tissue would also achieve those results, one of skill in the relevant art at the time this application was filed would have expected that bone marrow stromal cells producing an

angiogenic factor could be easily produced as taught by Applicant using routine methods known in the art, could be delivered intramyocardially to normal tissue adjacent to ischemic tissue in a diseased or ischemic heart as taught by Applicant, and would stimulate collateral blood vessel formation, induce angiogenesis and improve contractile function, and to a higher degree than would be achieved by delivering the cells to ischemic tissue rather than normal tissue. As such, one of skill in the art with Applicant's specification in hand and using the knowledge available to such a person at the time of filing, could make and use the invention without resorting to undue experimentation. Therefore, Applicant has satisfied the requirements of 35 U.S.C. 112, first paragraph.

In view of the foregoing remarks and amendments to the claims, applicant requests that the Examiner reconsider and withdraw the rejection of the claims under 35 U.S.C. 112, first paragraph for purported lack of enablement.

Applicant believes no additional fees are due with this response. However, if a fee is due, please charge our Deposit Account No. 06-2375, under Order No. WO-BSX 236 US2/10409073 from which the undersigned is authorized to draw.

Dated: September 13, 2006

Respectfully submitted,

By Mary Anne Schofield

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Human Umbilical Cord Blood Mononuclear Cells for the Treatment of Acute Myocardial Infarction

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Cell transplantation is a new treatment to improve cardiac function in hearts that have been damaged by myocardial infarction. We have investigated the use of human umbilical cord blood mononuclear progenitor cells (HUCBC) for the treatment of acute myocardial infarction. The control group consisted of 24 normal rats with no interventions. The infarct + vehicle group consisted of 33 rats that underwent left anterior descending coronary artery (LAD) ligation and after 1 h were given Isolyte in the border of the infarction. The infarct + HUCBC group consisted of 38 rats that underwent LAD ligation and after 1 h were given 10^6 HUCBC in Isolyte directly into the infarct border. Immunosuppression was not given to any rat. Measurements of left ventricular (LV) ejection fraction, LV pressure, dP/dt, and infarct size were determined at baseline and 1, 2, 3, and 4 months. The ejection fraction in the controls decreased from 88 ± 3% to 78 ± 4% at 4 months ($p = 0.03$) as a result of normal aging. Following infarction in the infarct + vehicle group, the ejection fraction decreased from 87 ± 4% to 51 ± 3% between 1 and 4 months ($p < 0.01$). In contrast, the ejection fraction of the infarcted + HUCBC-treated rat hearts decreased from 87 ± 4% to 63 ± 3% at 1 month, but progressively increased to 69 ± 6% at 3 and 4 months, which was different from infarct + vehicle group rats ($p < 0.02$) but similar to the controls. At 4 months, anteroseptal wall thickening in infarct + HUCBC group was 57.9 ± 11.6%, which was nearly identical to the control anteroseptal thickening of 59.2 ± 8.9%, but was significantly greater than the infarct + vehicle group, which was 27.8 ± 7% ($p < 0.02$). dP/dt_{max} increased by 130% in controls with 5.0 μg of phenylephrine (PE)/min ($p < 0.001$). In the infarct + vehicle group, dP/dt_{max} increased by 91% with PE ($p = 0.01$). In contrast, in the infarct + HUCBC group, dP/dt_{max} increased with PE by 182% ($p < 0.001$), which was significantly greater than the increase in dP/dt_{max} in the infarct + vehicle group ($p = 0.03$) and similar to the increase in the controls. Infarct sizes in the infarct + HUCBC group were smaller than the infarct + vehicle group and averaged 3.0 ± 2.8% for the infarct + HUCBC group versus 22.1 ± 5.6% for infarct + vehicle group at 3 months ($p < 0.01$); at 4 months they averaged 9.2 ± 2.0% for infarct + HUCBC group versus 40.0 ± 9.2% for the infarct + vehicle group ($p < 0.001$). The present experiments demonstrate that HUCBC substantially reduce infarction size in rats without requirements for immunosuppression. As a consequence, LV function measurements, determined by LV ejection fraction, wall thickening, and dP/dt, are significantly greater than the same measurements in rats with untreated infarctions.

Key words: Umbilical cord blood cells; Stem cells; Acute myocardial infarction; Infarct size; Left ventricular (LV) function; Ejection fraction; dP/dt

INTRODUCTION

Cell transplantation is a new treatment to improve cardiac function in hearts that have been damaged by myocardial infarction. Embryonic stem cells (21), fetal liver cells (29), skeletal myoblasts (14,22,23,53), and bone marrow stem cells (40,55,56) have been injected into myocardium to repair damaged hearts and improve heart function. Skeletal myoblasts or bone marrow stem cells have been predominantly used for cell transplantation for the treatment of myocardial infarction. Skeletal myoblasts, which constitute 4–8% of mammalian skeletal muscle, can differentiate into myotubes that express β-myosin heavy chain protein, which is characteristic of heart muscle (22,23,53). Myoblast transplantation into damaged hearts can increase left ventricular (LV) systolic and diastolic function (14,22,23,53). The improvement in LV function appears to be linearly related to the number of myoblasts transplanted (14). However, skeletal myoblasts must be propagated in culture for days or

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weeks prior to heart transplantation (23). Furthermore, formation of intercalated discs between donor skeletal myoblasts and host cardiomyocytes does not uniformly occur and does not persist when it does occur. Therefore, islands of skeletal muscle can form in the heart and cause electrical reentry during ventricular depolarization and cardiac arrhythmias (36).

Bone marrow stem cells are an alternative to skeletal myoblasts for cell transplantation. These cells have been either mobilized with cytokine or drug treatment from bone marrow into the systemic circulation or directly aspirated from bone marrow and injected into infarcted myocardium or into the circulation. These stem cells, by themselves or in combination with trophic factors, can propagate in the myocardium, express cardiac-specific proteins (β-myosin heavy chain, troponin, phospholamban), attach by gap junction proteins and electrically couple with neighboring host cardiomyocytes, and regenerate cardiomyocytes in infarcted myocardium (2,4, 40,41,51,55,56). Infarct size may be decreased by as much as 40% (41). In damaged hearts, these stem cells can also result in angiogenesis and neovascularization (13,18,26,27,41) and a decrease in myocardial perfusion defects measured by tomographic heart perfusion scans (3,42). Consequently, bone marrow stem cells can increase LV systolic pressure and wall thickness, decrease LV chamber size, and increase the ejection fraction in damaged hearts in both research animals and patients (3,13,18,26,27,42,51,56,62). However, between 150 and 1000 ml of bone marrow may be required to isolate adequate numbers of bone marrow stem cells (44). The stem cells expand slowly in culture, and expansion and purification of these cells may suppress their homing capacity and limit their effectiveness (18). For these reasons, autologus bone marrow stem cells are not immediately available for the treatment of acute myocardial infarction patients. Furthermore, transplantation of allogeneic hematopoietic and/or mesenchymal bone marrow stem cells commonly requires concomitant immunosuppression of the host (17,24,27,55).

Recognizing the limitations of skeletal myoblasts and bone marrow stem cells, we have investigated the use of human umbilical cord blood mononuclear progenitor cells (HUCBC) for the treatment of acute myocardial infarction. HUCBC contain hematopoietic and mesenchymal progenitor cells (6,7) that can potentially transdifferentiate into capillaries and cardiomyocytes. The total content of hematopoietic progenitor cells in umbilical cord blood equals or exceeds that present in bone marrow (6,7). The highly proliferative potential HUCBC are reported to be eightfold greater than similar cells in bone marrow and HUCBC express hematopoietic markers such as CD34 and CD14 (6,9,32). Human umbilical cord blood can contain four times as many $CD34^+$ cells as bone marrow, and the $CD34^+$ cells can differentiate into capillary endothelium (39). The immunotype and functional properties displayed by human cord blood mesenchymal cells closely resembles the characteristics assigned to bone marrow mesenchymal progenitor cells. Because HUCBC are readily accessible and can be cryopreserved for 15 or more years with recovery of 60–90% viable cells (8), these cells are available for the treatment of acute myocardial infarction.

The present report demonstrates the feasibility and efficacy of HUCBC transplantation in the treatment of acute myocardial infarction in the rat.

MATERIALS AND METHODS

Ninety-five male Sprague-Dawley rats, weighing 250–350 g, were housed in a temperature-controlled environment with free access to food and water. Infarction was produced in rats by ligation of the left anterior descending coronary artery (LAD). The rats were divided into three groups. The control group consisted of 24 normal rats with no interventions. The infarct + vehicle group consisted of 33 rats that underwent LAD ligation and, after 1 h, 0.2–0.5 ml Isolyte S, pH 7.4, was injected into the border of the infarction. The infarct + HUCBC group consisted of 38 rats that underwent LAD ligation and after 1 h were given 10^6 HUCBC in 0.2–0.5 ml Isolyte S, pH 7.4, in the border of the infarction. Immunosuppressive drugs were not given to rats that received HUCBC. The USF Institutional Review and Animal Use Committees approved all our protocols and procedures.

Cell Preparation

Cryopreserved (−196°C) mononuclear fractions of HUCBC were donated to us by a human cord cell blood bank (Saneron CCEL Therapeutics, Inc.). Maternal blood was examined for HIV, HTLV, hepatitis, syphilis, and cytomegalovirus and the cord blood was rejected if any test was positive. Cryopreserved HUCBC were thawed rapidly at 37°C and transferred into a centrifuge tube containing Isolyte S, pH 7.4 (B Braun McGaw Pharmaceuticals). The cells were extensively washed, centrifuged at 1000 rpm for 7 min, and the supernatant discarded. The HUCBC were labeled with fluorescein (FITC) conjugated to cholera toxin and then were extensively washed (16,49). The HUCBC viability was 75–85% using the Trypan blue dye exclusion method. HUCBC volumes were adjusted to 1 million viable mononuclear cells/0.2–0.5 ml Isolyte S, pH 7.4. All cells were injected into rat myocardium within 4 h of preparation.

Surgery

The rats were anesthetized with 30–90 mg/kg of ketamine and 2–9 mg/kg of xylazine, IP, intubated with polyethylene tubing, and mechanically ventilated (Har-

vard Apparatus) with room air. The rats were placed in the right lateral decubitus position on a heating pad. A thoracotomy was performed, the pericardium was opened, and the LAD was ligated 3 mm below its origin with 4-0 silk suture. Myocardial infarction was confirmed by anterior wall blanching and akinesis, and ST segment elevation on the electrocardiogram. One hour after acute infarction, each infarct + HUCBC rat received an intramyocardial injection of 10^6 HUCBC in the border of the infarction. Each infarct + vehicle group rat received only Isolyte S, pH 7.4, in the border of the infarction. The rats' lungs were then reinflated and the chest wall was closed. Normal saline was given to replace blood loss. The rats were then continuously monitored for 2 h. Buprenorphine (0.1–0.5 mg/kg, SC) was given for analgesia after surgery.

Echocardiography

Transthoracic echocardiograms were obtained on all rats. This technique is accurate and reproducible in normal rats and rats with infarction (30). An echocardiographic system (Siemens/Acuson) with a 7.5 MHz transducer was used. This system has a resolution of <0.5 mm (30). Rats were lightly anesthetized with phenobarbital (30–40 mg/kg, IP). 2D and M mode echocardiograms were obtained at the level of the papillary muscles and were recorded on Polaroid film and videotape. A minimum of three to five cardiac cycles were measured and the values averaged for each rat. A physician and a sonographer, who were unaware of the rats' category, analyzed the images. Left ventricular volumes were determined from the formula: $EDV = 7.0/(2.4 + LVDd) \times LVDd^3$ and $ESV = 7.0/(2.4 \pm LVDs) \times LVDs^3$, where EDV and ESV are end-diastolic and end-systolic volumes and D is the ventricular diameter in diastole (d) and systole (s) (30). Left ventricular ejection fraction was then determined from the EDV – ESV/EDV. Percent anteroseptal thickening was determined from the formula IVSs – IVSd/IVSd × 100, where IVS is the interventricular septum in systole (s) and diastole (d) (30).

Hemodynamic Studies

Rats from each group were lightly anesthetized with phenobarbital (30–40 mg/kg, IP), so that spontaneous respirations were maintained. The left femoral vein was cannulated with polyethylene tubing (Clay Adams). The tubing was connected to a microinfusion pump (Fischer Scientific) for phenylephrine (PE) administration. A pressure tipped catheter transducer (SPR-671, Millar Instruments) was electrically calibrated and then inserted either through the right carotid artery or transthoracically into the LV for pressure measurements. The distal end of the transducer was connected to a direct writing oscillograph (Gould TA 5000). Each animal was allowed to stabilize for 10–20 min prior to LV pressure and heart rate recordings. Measurements of LV pressure, the maximum rate of pressure rise (dP/dt_{max}) and fall (dP/dt_{min}), and heart rate were then determined from 1000 consecutive waveforms. Thereafter, PE, in concentrations of 0.5–5.0 μg/min, was infused into the left femoral vein and LV pressure, dP/dt_{max}, dP/dt_{min}, and heart rate were recorded after these measurements reached a plateau with each PE dose. At the termination of the experiment, each animal was given a lethal injection of phenobarbital.

Infarct Size

Hearts were harvested from randomly selected rats from the control group ($n = 4$), the infarct + vehicle group ($n = 16$), and the infarct + HUCBC group ($n = 24$) at 1, 2, 3, and 4 months after study initiation. Each heart was placed in saline (4°C) and then cut from apex to base in 2-mm slices. Each slice was placed in 1% triphenyl tetrazolium at pH 7.4 and 37°C for 40 min. The slices were rinsed with cold saline and fixed in 10% formalin. Each heart slice was then photographed with a digital camera and the images transferred to a workstation. The area of infarction and the area of normal ventricle were determined by software analyses (Image Pro Plus, Media Cybernetics and NIH image analysis software). The areas for each slice were then summed to determine total infarct area and the total area of the ventricles for each heart. Infarct size was then expressed as infarct area divided by total ventricular muscle area. Measurements were done in duplicate and the results averaged.

Histopathology

Two pathologists, who were unaware of the treatment for each rat, examined heart tissue sections from randomly selected rats from each group. Myocardial tissue was stained with hematoxylin and eosin (H&E), trichrome, and also pontamine sky blue to minimize autofluorescence. The tissue slides were examined with Zeiss and Olympus microscopes using rhodamine, fluorescein, or DAPI filters. Selected tissue sections were digitized and studied on a workstation.

Statistical Analysis

All results are expressed as the mean ± SEM. Differences among groups were tested by analyses of variance (ANOVA). The difference between two groups was tested by a Student's *t*-test. Multiple comparisons between groups were performed in our analyses. Consequently, the Bonferroni modification of the *t*-test was used for repeated statistical analyses. A value of $p < 0.05$ was judged to be significant.

RESULTS

Echocardiography Measurements

Echocardiograms were performed in each group at baseline and at 1, 2, 3, and 4 months. Figure 1 shows the LV ejection fraction measurements for each group of rats. The ejection fraction in controls was 88 ± 3% at baseline and decreased to 78 ± 4% at 4 months (p = 0.03) due to normal aging. Following myocardial infarction in the infarct + vehicle group, the ejection fraction decreased significantly from the baseline of 87 ± 4% to 51 ± 3% between 1 and 4 months ($p < 0.01$). In contrast, the LV ejection fraction in the infarct + HUCBC group decreased from the baseline of 87 ± 4% to 63 ± 3% at 1 month, but progressively increased to 69 ± 6% at 3 and 4 months. These measurements were significantly different from the infarct + vehicle group rats at 3 and 4 months ($p < 0.02$) but were similar to the control rats at 4 months.

Figure 2 shows representative 2D and M mode echocardiograms from each rat group 4 months after acute infarction. In the infarct + vehicle group rat (Fig. 2B), the LV cavity was dilated and the anteroseptal wall was akinetic. In contrast, in the infarct + HUCBC group rats (Fig. 2C), the LV cavity was normal in size and the anteroseptal wall was nearly normal. The anteroseptal wall thickening in the infarct + HUCBC group rats was greater than the infarct + vehicle group rats during the 4 months of observation. At 4 months after infarction, the percent anteroseptal wall thickening in the infarct + HUCBC group rats was 57.9 ± 11.6%, which was nearly identical to the control rat anteroseptal thickening of 59.2 ± 8.9 % but was significantly greater than the anteroseptal thickening in the infarct + vehicle rats, which was only 27.8 ± 7% ($p < 0.02$).

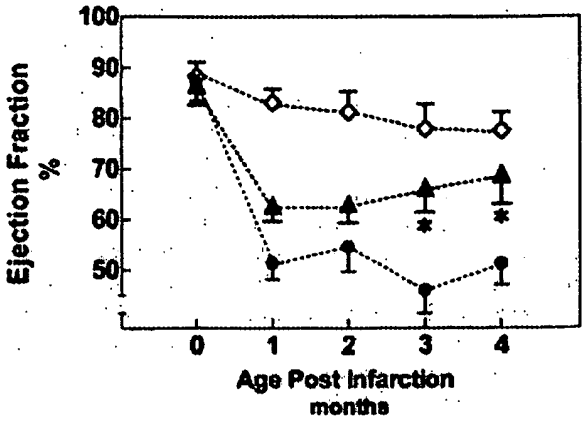

Figure 1. Mean echocardiographic short axis LV ejection fraction (%) for controls (◇), infarct + vehicle group (●), and infarct + HUCBC group (▲) at baseline (0) and at 1, 2, 3, and 4 months. The ejection fraction in the infarct + HUCBC group was significantly greater (*$p < 0.02$) than in the infarct + vehicle group at 3 and 4 months.

Hemodynamic Measurements

Left ventricular dP/dt measurements were performed prior to and during IV PE (0.5–5 μg/min), to rats from the control group ($n = 9$), the infarct + vehicle group ($n = 11$), and the infarct + HUCBC group ($n = 23$) (Fig. 3A). The dP/dt_{max}, an index of myocardial contractility, increased by a mean value of 130% in the controls from a normal value of 5667 ± 1155 mmHg/s to 13044 ± 1225 mmHg/s during 5.0 μg PE/min ($p < 0.001$). In the infarct + vehicle group, the dP/dt_{max} increased by a mean value of 91% from 3955 ± 803 to 7534 ± 941 mmHg/s ($p = 0.01$). In contrast, in the infarct + HUCBC group

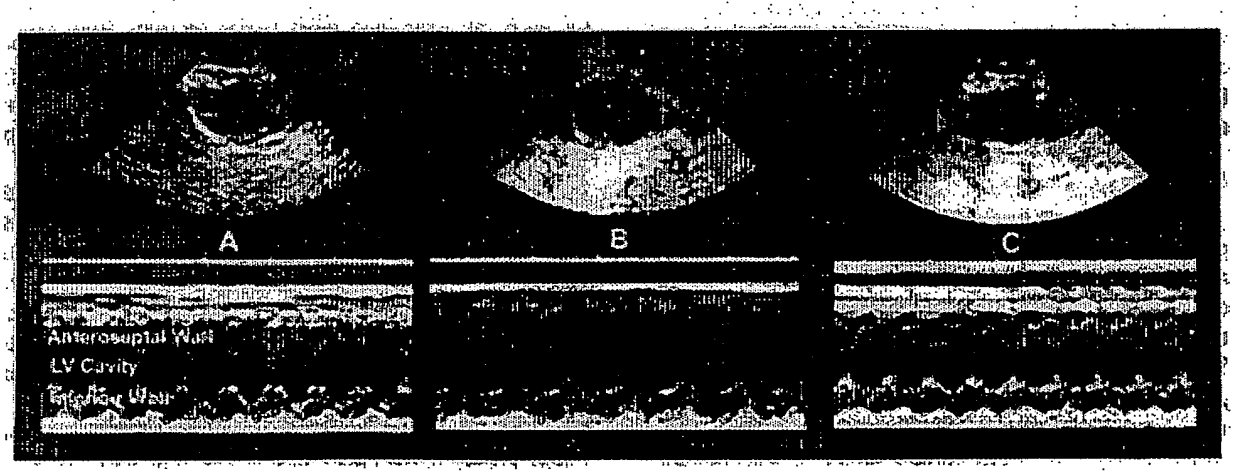

Figure 2. 2D and M-mode echocardiograms of representative rats from the control group (A), infarct + vehicle group (B), and infarct + HUCBC group (C) at 4 months. Short axis echocardiograms were taken at the level of papillary muscle heads. In the infarct + vehicle group rat (B), the anteroseptal wall is akinetic and the LV cavity is dilated.

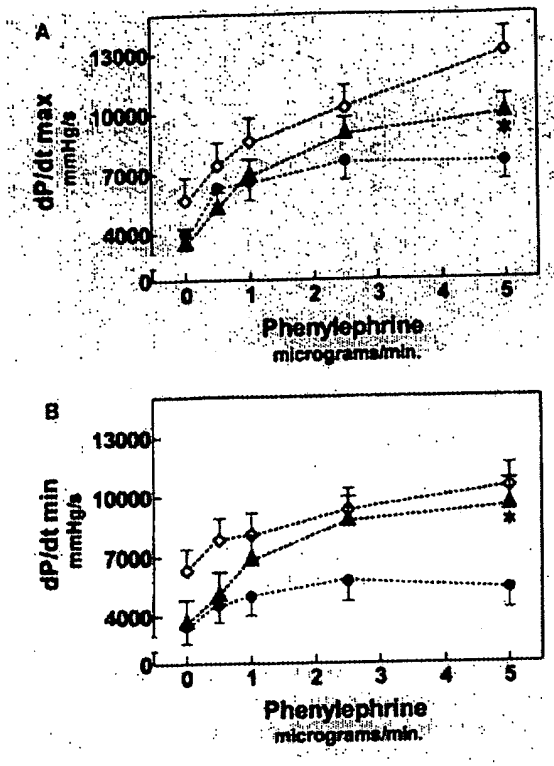

Figure 3. Mean dP/dt_{max} (A) and dP/dt_{min} (B) measurements in controls (◇), infarct + vehicle group (●), and infarct + HUCBC group (▲) in response to IV phenylephrine (0.5–5.0 μg/min). In (A) the increase in dP/dt_{max} in the infarct + HUCBC group with 5.0 μg/min phenylephrine was greater than the increase in the infarct + vehicle group ($*p < 0.03$). In (B) the maximal increase in dP/dt_{min} was greater ($*p < 0.01$) than the increase in infarct + vehicle group and was similar to the increase in the control rats.

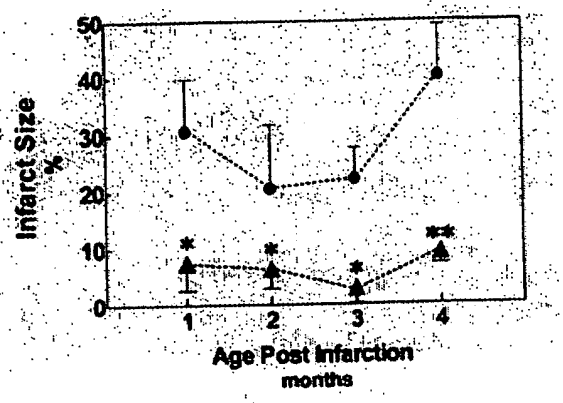

Figure 4. Mean infarct size (%) in the infarct + vehicle group (●) and the infarct + HUCBC group (▲) hearts at 1 ($n = 5$), 2 ($n = 6$), 3 ($n = 14$), and 4 ($n = 15$) months postinfarction. The infarct + HUCBC group infarctions were smaller than the infarct + vehicle group infarctions at each time point ($*p < 0.01$, $**p < 0.001$).

dP/dt_{max} increased by 182% from 3568 ± 701 to 10062 ± 784 mmHg/s during 5 μg PE/min ($p < 0.001$). The increase in dP/dt_{max} with 5.0 μg/min PE in the infarct + HUCBC group was significantly greater than the increase in the infarct + vehicle group ($p < 0.03$) and was similar to the increase in the controls.

Figure 3B shows the changes in dP/dt_{min}, an index of myocardial relaxation in response to PE. The dP/dt_{min} maximally increased by 66% in the controls from 6311 ± 1079 to 10452 ± 1144 mmHg/s with PE ($p < 0.02$). In the infarct + vehicle group, dP/dt_{min} increased by 54% from 3459 ± 872 to 5322 ± 1022 mmHg/s (p = NS). In

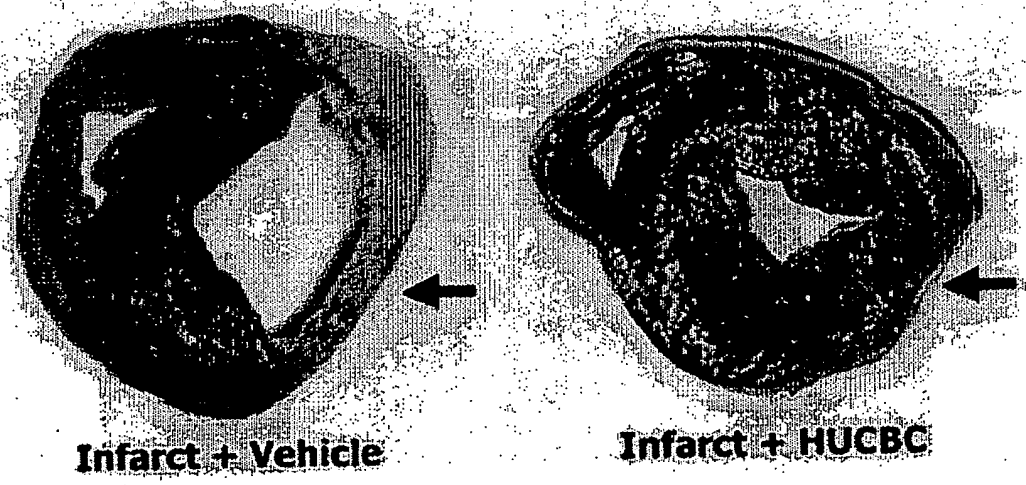

Figure 5. Transverse heart sections from a representative infarct + vehicle group and a infarct + HUCBC group rat taken at age 4 months postinfarction. The sections were stained with tetrazolium. Tetrazolium forms a red precipitate in the presence of intact dehydrogenase enzymes while areas of myocardial necrosis lack dehydrogenase activity and the infarcted heart tissue turns light pink or white. The arrows point to the infarcted myocardium in each myocardial section.

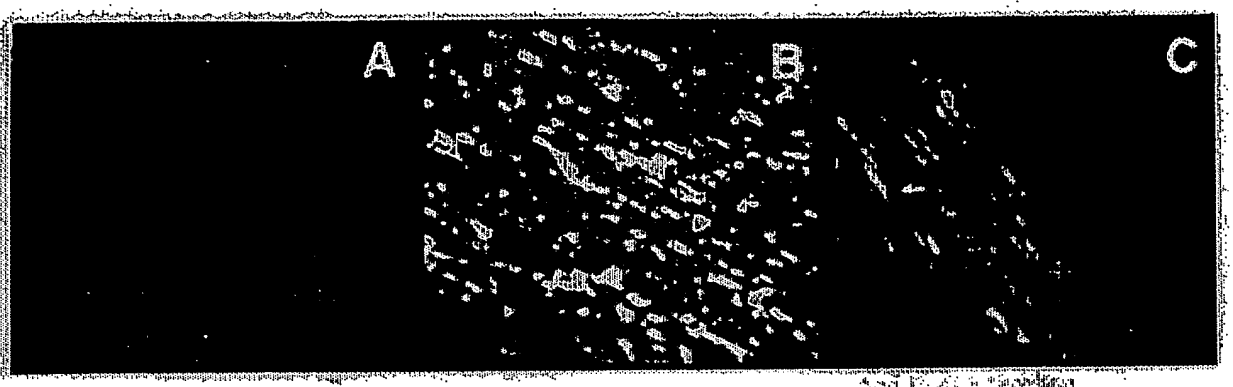

Figure 6. (A) Normal heart tissue with no autofluorescence. (B) HUCBC-treated heart tissue at 1 month in frozen section. The HUCBC are distal to the injection site and some cells have entered the infarction. (C) Treated heart at 3 months. The fluorescent HUCBC are aligned with the host cardiomyocytes. All sections were counterstained with pontamine sky blue to decrease autofluorescence.

contrast, in the infarct + HUCBC group, the dP/dt_{min} increased by 157% from 3748 ± 1064 to 9623 ± 1190 mmHg/s ($p < 0.01$). The increase in dP/dt_{min} in the infarct + HUCBC group exceeded the increase in the infarct + vehicle group for the 1–5 μg/min PE study. The maximal increase in dP/dt_{min} with PE in infarct + HUCBC group was greater ($p < 0.01$) than the increase in the infarct + vehicle group rats and was similar to the increase in the control rats (Fig. 3B).

Pathology

No infarction was present with tetrazolium staining in four control rats. In 16 infarct + vehicle-treated rats and in 24 infarct + HUCBC-treated rats the infarction

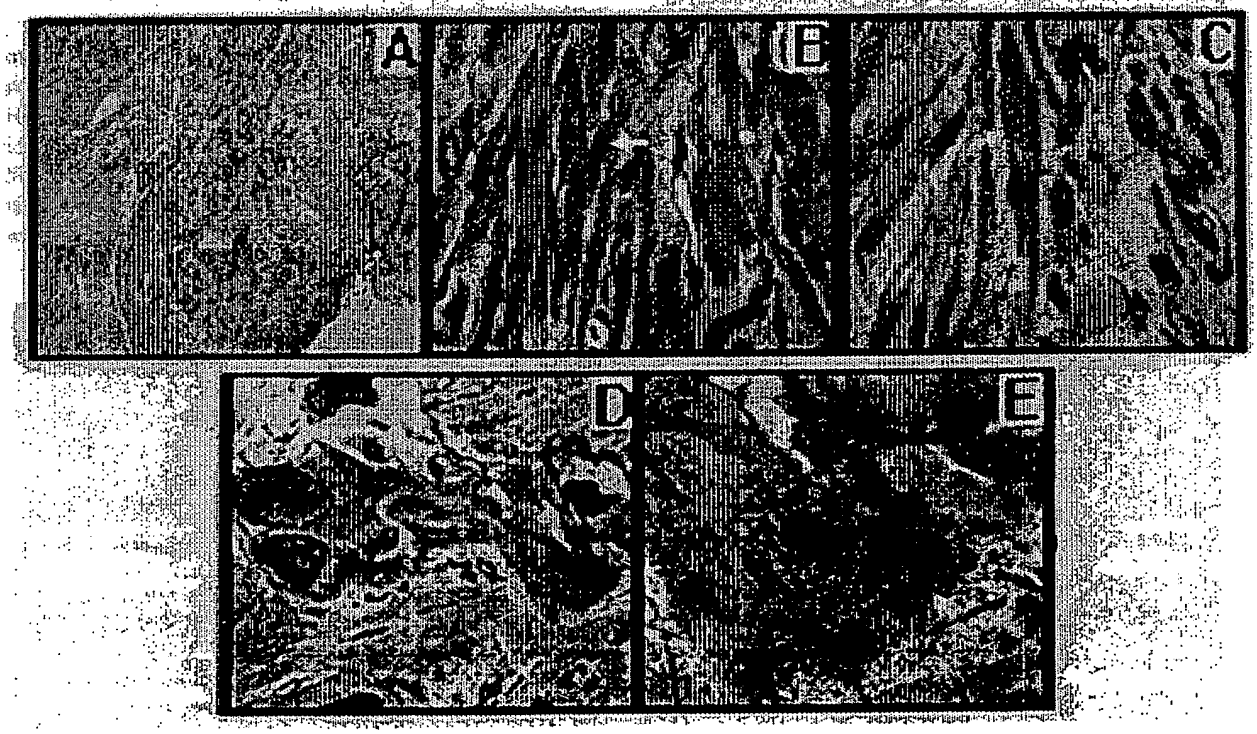

Figure 7. A treated infarct + HUCBC group heart. (A–C) H&E stains. (A) A layer of proliferating myoblasts and capillaries near the epicardial surface of the heart (×90 original magnification). (B) Proliferating myoblasts with centrally located hyperchromatic nuclei and striations in the cytoplasm. The fibers are nearly parallel to each other (×750 original magnification). (C) Mononucleated and binucleated myoblasts with abundant cytoplasm (×750 original magnification). (D and E) Electron micrographs of a myoblast with an intercalated disc adjoining normal myocytes in an infarcted heart treated with HUCBC.

area/ventricular area ratio was measured. The infarct sizes in the infarct + HUCBC group were significantly smaller than the infarct sizes in the infarct + vehicle group (Fig. 4). The differences in infarct size were most pronounced at 3 and 4 months after infarction. The infarct sizes at 3 months averaged 3.0 ± 2.8% for the infarct + HUCBC group versus 22.1 ± 5.6% for infarct + vehicle group ($p < 0.01$) and at 4 months averaged 9.2 ± 2.0% for infarct + HUCBC group versus 40.0 ± 9.2% for infarct + vehicle group ($p < 0.001$).

Figure 5 shows transverse tissue sections taken through the left and right ventricles of a representative infarct + vehicle rat heart and an infarct + HUCBC-treated rat heart stained with tetrazolium at 4 months after myocardial infarction. The infarct + vehicle rat heart has a densely scarred anterior wall, which encompasses >30% of the ventricular muscle and a dilated LV cavity. In contrast, the anterior wall infarction in the infarct + HUCBC rat heart encompassed only 9% of the ventricular muscle and the LV cavity is not dilated.

The HUCBC were labeled with fluorescein (FITC) prior to injection in the infarct + HUCBC group rats. Figure 6A shows normal heart tissue. Figure 6B and C shows the appearance of the HUCBC in infarct + HUCBC rat hearts at 1 (Fig. 6B) and 3 months (Fig. 6C) after infarction. Fluorescent HUCBC are aligned with the host cardiomyocytes at 3 months. Little or no autofluorescence occurred in the infarct + vehicle group or in controls hearts.

Figure 7A–C shows myocardial histological sections from a treated infarct + HUCBC heart stained with H&E. Figure 7A (90× original magnification) shows a layer of proliferating myoblasts and capillaries near the epicardial surface of the myocardium. Figure 7B (750× original magnification) shows proliferating myoblasts with centrally located hyperchromatic nuclei and striations in the cytoplasm. The fibers are nearly parallel to each other. Figure 7C (750× original magnification) shows mononucleated and binucleated myoblasts with abundant cytoplasm. Figure 7D and E shows electron micrographs of a HUCBC-treated infarct containing a myoblast with an intercalated disc adjoining normal myocytes. Proliferating myoblasts were not present in the infarct + vehicle hearts.

Despite the fact that the rats in infarct + HUCBC group did not receive immunosuppression, there was no histologic evidence of HUCBC rejection on H&E-stained heart tissue taken at 1, 2, 3, or 4 months after infarction.

DISCUSSION

The present experiments suggest that HUCBC can persist in rat infarcted myocardium without requirements for host immunosuppression. These cells produce a substantial reduction in acute myocardial infarction size in comparison with untreated infarcted hearts. As a consequence, LV function measured by LV ejection fraction and wall thickening and by dP/dt_{max} and dP/dt_{min} are significantly greater than the same measurements in rat hearts with untreated infarctions. The present experiments suggest that HUCBC are beneficial for limiting and/or repairing the myocardial damage due to acute infarction.

Human Umbilical Cord Blood Cells

Umbilical cord blood is potentially widely available for therapeutic purposes because 3.5 million births occur each year in the US. Nevertheless, umbilical cord blood is considered by many medical centers a waste product that is routinely discarded after childbirth. Umbilical cord blood contains long-term culture initiating cells that are capable of self-renewal and have the ability to generate multipotential, erythroid, and myeloid progenitor colonies (34,35). These cells generate almost a log more progeny than their bone marrow counterparts (19).

One milliliter of human umbilical cord blood contains approximately 8000 primitive erythroid progenitor cells, 13,000–24,000 myeloid progenitor cells, and between 1000 and 10,000 multipotent progenitor cells (1,57). The content of primitive hematopoietic progenitor cells in umbilical cord blood varies with gestational age. For example, $CD34^+$ progenitor cells comprise approximately 11% of small mononuclear cells at 17 weeks gestation but only approximately 1–2% by 38 weeks gestation (63). Moreover, progenitor cells in cord blood cycle slowly (G_0/G_1) in culture but respond to stem cell factor and other growth factors by traversing into the S phase of the cell cycle (57).

Umbilical cord $CD34^+$ cells, when cultured with interleukin-2, give rise to endothelial cells that express von Willebrand factor, CD31 (PECAM), CD54 (ICAM-1), and CD62 (E-selectin) (39). It is not currently known if these cells release vascular endothelial growth factor (VEGF) and angiopoietin I or differentiate into cardiomyocytes, which has been described for endothelial progenitor cells from bone marrow and peripheral blood (24).

While 70–80% of adult blood mononuclear cells are $CD3^+$, only approximately 50% of HUCBC are $CD3^+$ (46,47). Human umbilical cord blood lymphocytes are immature and produce fewer cytokines and immunoglobulins than adult lymphocytes (20,46,47,59). Consequently, little or no cytotoxic activity is generated with cord blood after primary, secondary, and tertiary allogeneic stimulation (47,60). Moreover, few HUCBC express class II HLA antigens (60). The diminished cytotoxicity and immaturity of HUCBC may explain the small incidence of graft versus host disease with cord blood transplantation in patients (60) and the absence of immunological reactions in our HUCBC-treated rats

during the 4 months of observation in the present experiments.

Umbilical cord blood also contains mesenchymal stem cells that express surface antigens SH2, SH3, SH4, α-smooth muscle actin, MAB 1470, CD13, CD29, and CD49e (12). Similar mesenchymal stem cells reside in the bone marrow and can differentiate into myogenic cells, express muscle specific proteins, and also action potentials (28,45,55). Recently, bone marrow mesenchymal stem cells have been given for treatment of acute myocardial infarction and have expressed cardiac-specific proteins, preserved left ventricular wall thickness, and attenuated contractile dysfunction (51,56).

More than 60–90% of nucleated cells can be recovered from cord blood that has been frozen for 15 or more years (8). Consequently, blood banks for umbilical cord blood storage and transplantation have been established in the US and Europe. More than 1500 cord blood cell transplants have been performed in the treatment of patients with Fanconi anemia, aplastic anemia, β-thalassemia, severe combined immune deficiency, X-linked lymphoproliferative syndrome, Hurler syndrome, Hunter's syndrome, Wiskott-Aldrich syndrome, acute lymphoid leukemia, acute myeloid leukemia, chronic myeloid leukemia, juvenile chronic myeloid leukemia, myelodysplastic syndrome, and neuroblastoma (6,15). The fact that HUCBC are beneficial in the treatment of a variety of different human metabolic and hematological diseases suggests that HUCBC transplantation should be investigated for the treatment of other clinical disorders.

Mechanisms Whereby HUCBC Improve Heart Function

Several studies suggest that HUCBC can change phenotype. Human umbilical vein endothelial cells have been reported to differentiate into cardiomyocytes and $CD34^+$ HUCBC can differentiate into skeletal muscle (11,43). Umbilical cord cells have also been used for tissue engineering of heart valves and can express α-smooth muscle actin, desmin, and vimentin (25). In rats with stroke or brain trauma and in neonatal rats, HUCBC express proteins in the brain that are phenotypic of neural cells and have the capacity to significantly limit brain ischemic and traumatic damage (10,31,64). In addition, HUCBC, when treated in vitro with nerve growth factor and retinoic acid, or basic fibroblast growth factor and human epidermal growth factor, can express specific neuronal and glial proteins (48). These studies suggest that HUCBC are capable of expressing specific cell proteins and also transdifferentiating into cells that may be beneficial in the repair of ischemic tissue.

An alternative mechanism whereby HUCBC may be beneficial in ischemic heart tissue is by angiogenesis. Endothelial progenitor cells are normal components of umbilical cord blood that can promote angiogenesis by releasing proangiogenic molecules such as vascular endothelial growth factor (37,39,43). These cells also express cell surface molecules such as KDR, Tie2/Tek, and VE-cadherin, which are expressed by endothelial cells during vasculogenesis (37). In addition, $CD34^+$ HUCBC can integrate into the walls of blood vessels in the periphery of injured tissue and can increase capillary density in ischemic muscles (10,31,37,39,43). Therefore, HUCBC may limit myocardial infarction size by promoting angiogenesis in the myocardium.

The beneficial effect of HUCBC transplantation in ischemic and infarcted myocardium may also be due to release of growth factors, cytokines, nitric oxide, or other factors that limit organ damage and remodeling that occurs after infarction. In this regard, the intravenous administration of HUCBC into rats with stroke increases the brain concentrations of growth and neurotrophic factors that facilitate neuroprotection, even though the HUCBC may not directly enter the central nervous system (5). HUCBC are also capable of producing cytokines such as interleukin-1β, granulocyte macrophage colony stimulating factor, and interleukin-3, which facilitate proliferation and expansion of progenitor cells, and integrins, which may be important in homing cells to ischemic sites (10,50,61). Stem cells also appear capable of expressing natriuretic peptides that may limit acute edema formation after major organ infarction or trauma, and these cells also express vascular, fibroblastic, hepatic, and insulin-like growth factors that may affect remodeling (4,33,52).

In the present study, the presence of myoblasts in the myocardial infarction in the treated rat hearts suggests that HUCBC are either able to transdifferentiate into myoblasts or stimulate the transdifferentiation of native progenitor cells present in the rat myocardium. The precise mechanisms whereby human umbilical cord blood mononuclear cells minimize ischemic damage in acute myocardial infarction are not defined in the present study but are currently being investigated in our laboratory.

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This Review is part of a thematic series on Stem Cells, which includes the following articles:

Differentiation of Pluripotent Embryonic Stem Cells Into Cardiomyocytes
Derivation and Potential Applications of Human Embryonic Stem Cells
Stem Cells for Myocardial Regeneration
Myocyte Death, Growth, and Regeneration in Cardiac Hypertrophy and Failure
Neural Stem Cells: An Overview
Mesenchymal Stem Cells and Their Potential as Cardiac Therapeutics

Therapeutics and Use of Stem Cells — *Toren Finkel, Roberto Bolli, Editors*

Mesenchymal Stem Cells and Their Potential as Cardiac Therapeutics

Mark F. Pittenger, Bradley J. Martin

Abstract—Mesenchymal stem cells (MSCs) represent a stem cell population present in adult tissues that can be isolated, expanded in culture, and characterized in vitro and in vivo. MSCs differentiate readily into chondrocytes, adipocytes, osteocytes, and they can support hematopoietic stem cells or embryonic stem cells in culture. Evidence suggests MSCs can also express phenotypic characteristics of endothelial, neural, smooth muscle, skeletal myoblasts, and cardiac myocyte cells. When introduced into the infarcted heart, MSCs prevent deleterious remodeling and improve recovery, although further understanding of MSC differentiation in the cardiac scar tissue is still needed. MSCs have been injected directly into the infarct, or they have been administered intravenously and seen to home to the site of injury. Examination of the interaction of allogeneic MSCs with cells of the immune system indicates little rejection by T cells. Persistence of allogeneic MSCs in vivo suggests their potential "off the shelf" therapeutic use for multiple recipients. Clinical use of cultured human MSCs (hMSCs) has begun for cancer patients, and recipients have received autologous or allogeneic MSCs. Research continues to support the desirable traits of MSCs for development of cellular therapeutics for many tissues, including the cardiovascular system. In summary, hMSCs isolated from adult bone marrow provide an excellent model for development of stem cell therapeutics, and their potential use in the cardiovascular system is currently under investigation in the laboratory and clinical settings. (*Circ Res.* 2004;95:9-20.)

Key Words: cardiomyoplasty ■ mesenchymal ■ cell therapy ■ stem cell

The last 5 years have witnessed an explosion of new reports on human stem cells isolated from a variety of sources including embryonic, fetal, and adult tissues. The recognition that stem cells are found in many adult tissues and that these cells may lend themselves to tissue repair or regeneration has been particularly exciting. But where do stem cells originate, how do they maintain themselves, how is their fate determined, and can stem cells move from one tissue to another to provide regenerative cells? These questions quickly give rise to other questions centered around whether we can manipulate stem cells: Can they be isolated for study or only maintain their stem cell abilities in their in vivo niches? Can they be culture expanded or does this change their identity, characteristics, and abilities? Can they be delivered from exogenous sources to repair tissue, or can their numbers be boosted in situ? How may they be used to treat damaged and diseased tissues? In this review, we focused on the human mesenchymal stem cells (hMSCs)

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isolated from adult bone marrow and used by many investigators. Plating studies indicate MSCs are present as a rare population of cells in bone marrow, representing perhaps 0.001% to 0.01% of the nucleated cells, ≈10-fold less abundant than hematopoietic stem cells (HSCs), but MSCs can be readily grown in culture. Although the current knowledge of MSCs will further evolve, it is possible to move forward and use these isolated multipotential cells to ask critical questions that could not be addressed previously. MSCs offer a very useful compromise among the many different stem cells now described in terms of the ease of accessibility, handling, and multilineage potential, and their investigation illustrates how much has been learned and how much there is to understand still. Importantly, cultured MSCs have already been infused in humans for safety and early clinical testing for support of bone marrow transplantation, treatment for osteogenesis imperfecta, and glycogen storage disease, fields where there are not many therapeutic options.[1–5a]

Enthusiasm for cell therapy for the injured heart has already reached the clinical setting, with physicians in several countries involved in clinical trials using several cell populations. Results from these early reports suggest new therapies may come from the use of progenitor cells such as myoblasts[6] or the stem cells found in bone marrow.[7,8] Recognizing there are stem/progenitor cells in marrow including the MSC, the HSC, as well as those of the endothelial system,[9] it will be necessary to sort out the relative contributions of different cell types. Purified MSCs have tested well in animal cardiac models and are now being readied to head into the clinic.

There has long been evidence for a cellular basis for tissue damage repair, and there has been debate over whether the cells involved in tissue repair were of local origin or arrive by way of circulation. With the search for cells that would allow for survival after radiation exposure during the 1940s and 1950s, it became apparent that an HSC function could be found in the spleen and bone marrow.[10,11] HSCs were elusive, difficult to isolate and grow in culture (and this remains true today). During this time period, studies also suggested that bone marrow, when transplanted to an ectopic site, could form new bone tissue.[12] Recognition that there might be particular isolatable cells present in bone marrow capable of becoming osteoblasts and responsible for ectopic bone formation was provided by Friedenstein et al using a guinea pig model.[13]

Research on bone-forming cells of marrow was continued by many investigators.[14,15] There was also recognition of a bone marrow connection between bone-forming and adipose-forming cells.[16] During this period, bone marrow stroma continued to be seen as providing the essential microenvironment for HSC survival, and marrow cell studies of stromal function were performed.[17,18] The appreciation that there may be an MSC capable of differentiating to many or all mesenchymal lineages developed in part as a result of understanding lineage diagrams drawn for HSCs.[14,19] Isolation of the hMSC was undertaken by Caplan in the late 1980s and involved a rethinking of the isolation procedures and means to assay for the presence of the rare MSCs in small aspirates of bone marrow.[19] An important contribution was the use of an osteoconductive ceramic matrix for implantation studies that would faithfully reveal the cell osteochondral potential of the human cells cultured ex vivo when implanted in immunocompromised mice. This in vivo assay allowed them to select appropriate culture conditions for isolation and expansion of the hMSCs from bone marrow.[23] Of particular importance was attention to the lot of FBS selected for MSC growth.[20] Because we do not understand the full growth requirements of hMSCs (or other stem cells), a serum-free medium with defined growth factor composition has not been fully developed yet.

MSCs or Mesenchymal Progenitor Cells

The appropriately selected cell from adult tissue, which can be greatly and efficiently expanded in culture and can differentiate to several specific mesenchymal cell lineages, is a proper stem cell, an MSC. The hMSC from bone marrow can be cloned and expanded in vitro ≥1 million-fold and retain the ability to differentiate to several mesenchymal lineages.[21–23] Researchers have not yet found conditions that allow continuous, indefinite hMSC growth, yet it is possible to produce billions of MSCs in vitro for cellular therapy from a modest bone marrow aspirate drawn through the skin. MSCs need to be expanded ex vivo because they apparently are very contact inhibited, and there is little evidence of in vivo expansion as MSCs labeled with membrane dyes, that would be diluted and undetected from dividing cells after ≈3 divisions, are found months later even in repairing tissue (see Figure 5).

The current work with embryonic stem (ES) cells and neural stem cells is hindered by difficulties in producing enough cells in a reproducible manner to contemplate therapeutic development. The hMSCs, with their attributes of (1) ease of isolation, (2) high expansion potential, (3) genetic stability, (4) reproducible attributes from isolate to isolate, (5) reproducible characteristics in widely dispersed laboratories, (6) compatibility with tissue engineering principles, and (7) potential to enhance repair in many vital tissues, may be the current preferred stem cell model for cellular therapeutic development.[21–23,30,41–43,45,49]

Whether MSCs are capable of transdifferentiation to ectodermal lineages awaits full evidence, but results from RT-PCR and microarray analysis show that MSCs express genes normally thought to be expressed in neurogenic, ectoderm-derived tissue. Several studies have demonstrated the expression of neural genes by MSCs and improved results from MSC implantation at the sites of spinal or intracranial injury.[24–27] Whether such improvements come from MSCs producing growth factors and cytokines, MSC differentiation, or MSCs interacting with other cell types at the site of injury is still under investigation.

Sources and Characteristics of hMSCs

The fibroblastic cells from bone marrow are sometimes generically termed bone marrow stromal cells, but not all stromal cells from bone marrow are multipotent.[21,28,29] Researchers have described a variety of cells from bone marrow and given them different names, and at times, a particular

Figure 1. hMSCs in culture appear fibroblastic and homogeneous in size and morphology by second passage. Flow cytometry applying fluoresceinated antibodies was used to determine surface molecules present in the expanded cell population, and both positive and negative surface molecules are listed.

hMSC Surface Markers

Positive	Negative
CD13, CD29, CD44, CD49a, b, c, e, f, CD51, CD54, CD58, CD71, CD73, CD90, CD102, CD105, CD106, CDw119, CD120a, CD120b, CD123, CD124, CD126, CD127, CD140a, CD166, P75, TGFb1R, TGFbIIR, HLA- A,B,C, SSEA-3, SSEA-4, D7	CD3, CD4, CD6, CD9, CD10, CD11a, CD14, CD15, CD18, CD21, CD25, CD31, CD34, CD36, CD38, CD45*, CD49d, CD50, CD62E,L,S, CD80, CD86, CD95, CD117, CD133, SSEA-1

laboratory has changed names to refer to the same cells, leading to some nomenclature confusion. Most surface markers have been found inadequate as a means to identify stem cells because the putative marker(s) may also be found on nonstem cells, or a particular marker may only be expressed on a stem cell at a certain stage or under certain conditions, such as with CD34 on HSCs. Nevertheless, surface markers or other attributes are useful in characterizing the stem cell as isolated or cultured, and as a means to begin to understand its potential interactions with neighboring cells and the cell environment. A number of surface molecules on hMSCs are given in Figure 1 and, although extensive, are incomplete. Some variation in the surface molecules on hMSCs has been seen from laboratory to laboratory, and further work is necessary to understand whether these differences represent separate stem cell populations, different culture techniques, or means of analysis. MSCs from different species, studied by somewhat different methods, and in labs worldwide, have remarkably reproducible attributes.[21–23,42–45]

Adult bone marrow MSCs are thought to be largely quiescent, and the extent to which they proliferate in vivo is unknown. The initial expansion process is a result of those colony-forming units (the single cells that continue to divide to form clonal cell colonies). Although perhaps quiescent, bone marrow MSCs can divide rapidly once cell division begins, and this is seen best at very dilute plating density. Although differences in cell morphology and characteristics may initially exist, the MSC population becomes very homogeneous with time in culture and remains so for many passages. Cytogenetic testing of late passage–cultured hMSCs has not detected chromosomal abnormalities, and telomerase activity was also evident in these cultured late-passage hMSCs.[25]

Subpopulations of hMSCs

MSCs are not defined by their source, and even within a tissue such as bone marrow, there may be microenvironments wherein closely related or identical cells may express different surface molecules. For example, those MSCs isolated directly from bone marrow and tested for surface molecule expression will differ in some aspects from MSCs cultured for weeks in vitro. This includes CD45, which is considered a marker of hematopoietic lineages, yet it is identified on some MSCs from bone marrow at low levels and is quickly lost in culture.[30] Similarly, culture-expanded MSCs with slightly different characteristics may be derived depending on the culture methods used or the analysis methods. The Table compares the detected surface molecules on multipotential MSC-like cells from several labs, cultured in somewhat different conditions. Many of these isolated cells behaved similarly in in vitro differentiation experiments.

Prockop et al[31,32] have used the low-density plating methods of Friedenstein and isolated and studied the population of rapidly dividing cells from human bone marrow, termed recycling stem (RS) cells, as a subpopulation of MSCs. RS cells were termed RS-1 for the small agranular, rapidly dividing cells, and RS-2 for small, granular cells, whereas the more typical fibroblastic MSCs were considered mature MSCs in the cultures. RS cells divided very quickly when plated at low density and may yield 10^9 cells in 6 weeks.[33] Most recently, Prockop et al identified the Wnt inhibitor dickkopf-1 as an effector molecule for hMSC proliferation in vitro.[34]

Exciting recent findings have come from a series of articles from Verfaillie et al, who described marrow progenitor cells or multipotent adult progenitor cells (MAPCs).[35–38] The cultured cells have many of the attributes of MSCs, but they

Surface Antigen Comparison Among MSC-like Cells

Surface Antigen	MSCs[21]	MAPCs[38]	RS-1[33]	PLAs[43]	APCs[42]
CD9					+
CD10		−	−		+
CD11a,b	−		−		−
CD13	+	+		+	+
CD14	−		−	−	−
CD18 integrin β2	−				−
CD29	+			+	+
CD31 PECAM	−	−	+/−	−	
CD34	−	−	−	−	+
CD44	+		+	+	+
CD45	−*	−	−	−	−
CD49b integrin α2	+	+			
CD49d integrin α4	−			+	+
CD49e integrin α5	+		+		+
CD50 ICAM3	−	−			−
CD54 ICAM1	+				+
CD56 NCAM				−	−
CD62E E-selectin	−	−		−	−
CD71 transferrin rec	+		+	+	
CD73 SH-3	+			+	
CD90 Thy-1	+	+	+/−	+	
CD105 endoglin, SH-2	+			+	+
CD106 VCAM	+	−		−	+
CD117	−	−			
CD133	−	(+)	−	−	
CD166 ALCAM	+				+
Others					
β2 microglobulin	+	+			
Nestin	+			+	
p75	+			+	
HLA ABC	+	−	+/−		+
HLA DR	− Induc	−	−		−
SSEA-4	+	+			
TRK (A, B, C)	+		+		
Differentiation in vitro					
Osteo	+	+	+	+	+
Adipo	+	+	+	+	+
Chondro	+	+	+	+	
Neural	(+)			(+)	
Stromal	+	+			
Myoblast Sk	(+)	+		+	
Endothelial	(+)	+			

(+) indicates detection varied; *positive upon isolation.[30]

APCs indicates adipose progenitor cells; PLAs, processed liposuction aspirates; PECAM, platelet endothelial cell adhesion molecule; ALCAM, activated leukocyte cell adhesion molecule; ICAM, intercellular adhesion molecule; NCAM, neural cell adhesion molecule; VCAM, vascular cell adhesion molecule; HLA, human leukocyte antigen.

are reported to expand indefinitely and have lineage potentials that include extended ectodermal and endodermal cell types, making them more like ES cells. Additionally, they lack major histocompatibility complex (MHC) class I and class II on their surface, so presumably, they may be used allogeneically, although the lack of MHC I molecules may make them vulnerable to elimination by natural killer cells. Much of the published data are of cells of mouse origin, and it will be important to verify that the human MAPCs are as potent in these respects as the mouse MAPCs.[39,40]

It is possible to isolate MSCs from different mesenchymal tissues, and recently, it was shown that fibroblastic cells from adipose differentiate to mesenchymal lineages, and their characteristics and behavior are virtually indistinguishable from bone marrow–derived MSCs.[41–44] These cells have been termed adipose stromal cells, adipose progenitor cells, or processed liposuction aspirates.

Stem cells need to be functionally defined. Although there may be characteristics or surface markers that lead one to suspect a cell is a potential stem cell, the ability to produce daughter cells as well as differentiate to multiple phenotypes must be assayed. Small differences in surface markers may not be sufficient to distinguish types or subpopulations of stem cells. Functional comparison among isolated MSC populations is a better approach. In an effort to compare MSCs, adipose progenitor cells, and MAPCs prepared by protocols from different laboratories, Lodie et al performed an evaluation of their cellular characteristics and differentiation potential.[45] These authors found the cells produced by the different methods to be virtually indistinguishable. However, it is possible that a particular source of multipotential cells or a particular culture method may prove superior to other methods when cells are tested in vivo for the ability to repair a particular damaged tissue, and therefore, it is still useful to examine sources of stem cells and culture conditions.

There have been recognized differences between stem cells found in distinct species such as different in vitro requirements to culture HSCs from mice and man.[46,47] Similarly, MSCs can be isolated by a number of methods from different tissue sources, but their stem cell nature cannot be assured; it must be assayed. Recently, Simmons et al[18,48] reported the characterization of a subpopulation of marrow stromal cells selected by an antibody to an unknown surface marker, STRO-1. The selected cells from bone marrow have attributes of MSCs.[49]

Immune Responses and MSCs

Several laboratories have begun to characterize the interactions between MSCs and cells of the immune system. hMSCs have a number of surface molecules that would predict interaction with T cells. MHC I, Thy-1 (CD90), vascular cell adhesion molecule (CD106, VCAM), intercellular adhesion molecule 1 (ICAM-1) and ICAM-2, activated leukocyte cell adhesion molecule (CD166, ALCAM), lymphocyte functional antigen-3, and integrins α1 to α3, α5, α6, β1, β3, and β4 (CD49 a, b, c, e, and f) are surface molecules expressed on hMSCs that have cognate ligands on T cells.[21,28,50,51] Additionally, although not expressed at appreciable levels on hMSCs, MHC II expression is upregulated by incubating the cells with interferon-γ. However, the hMSCs lack the B7 costimulatory molecules CD80 and CD86, and these are not expressed after interferon treatment.[52,53]

Intuitively, one might expect allogeneic MSCs (allo-MSCs) would stimulate T cell proliferation and that donor MSCs would be recognized by responder T cells and rejected by a recipient host. However, experimental evidence indicates this may not be the case. MSCs have been shown to inhibit T cell proliferation in several laboratories.[52–55]

The interaction of T cells with other cell types can be studied in vitro. The mixed lymphocyte reaction (MLR) or exposure of peripheral blood mononuclear cells or isolated T cells to cells from another individual usually evokes a T-cell proliferative response that is measured conveniently by incorporation of [^{3}H]-thymidine into DNA during a several-day period. However, MSCs cultured with responder T cells do not generally cause T cell proliferation, and they usually reduce the response of responder T cells to other stimulator cells or nonspecific activators such as phytohemagglutinin. The lack of response is not because of T cell apoptosis or other deleterious effects because the T cells can be recovered and respond to other stimulators in the absence of MSCs. Tse et al described similar experiments including stimulating T cells with anti-CD28 and anti-CD3 antibodies before addition of MSCs.[53] The MSCs can inhibit the T cell proliferative response through a Transwell membrane insert, suggesting a soluble component is involved. Di Nicola et al[54] have shown that antibodies to hepatocyte growth factor and transforming growth factor β (TGF-β) restored the proliferative response, suggesting these factors are at least partially responsible. Le Blanc et al confirmed the MLR experiments and also demonstrated the dose-dependent inhibition of T cell proliferation, independent of the source of cultured MSCs.[55] Related studies in mice found MSCs inhibited the response of memory T cells to their antigen.[56]

It has been suggested that once stem cells begin to differentiate, they will express MHC II molecules and be rejected by T cells. However, Le Blanc et al caused MSCs to differentiate in vitro and then tested their interaction with T cells and found no increased antigenicity.[57] Although not all published, numerous animal studies have found the persistence of allo-MSCs in vivo, some of which are highlighted below.

Overall, MSCs appear to prevent maturation of T cells to their cognate antigen or other antigens by a direct cell–cell effect and a soluble factor or factors. Then it is possible that MSCs in close proximity can play a role in modulating the immune response of several effector immune cells (S. Aggarwal and M.F. Pittenger, submitted for publication). MSCs may serve a role to preserve progenitor or immature lymphocytes in the central bone marrow space while allowing for expansion elsewhere in the periphery as needed. These concepts will need further study in vivo and in vitro before we understand the direct and indirect effects MSCs can have on the immune response.

Several studies have used allo-MSCs in vivo, and experience suggests the allo-MSCs are not rejected and may have positive effects on engraftment of third-party cells or tissue.[58] Bartholomew et al tested the ability of MSCs to enhance skin engraftment in an allogeneic baboon model. In another study, MSCs were found after 9 months in multiple tissues in an irradiated baboon model of bone marrow transplantation, whether allogeneic or autologous baboon MSCs were used.[59]

Stem cell–based therapies could be very expensive to fully develop if they were based on autologous cells because it would be necessary to obtain a biopsy from the patient, expand the cells in culture, perform the required testing, and store retains before administering the therapy. Also, it is not clear whether each donor will produce sufficient stem cells of required potency at the needed time. An alternative approach is use of allogeneic stem cells. The advantages of allo-MSCs are many: the donor can be chosen ahead, qualified, and tested for absence of different disease organisms, the MSCs ready in advance so they are immediately available when needed by a patient.

Collectively, current studies on the interactions between MSCs and T cells support the potential use of allo-MSCs in cell therapy. Along with studies of allo-MSCs introduced into infarcted heart models and encouraging early clinical results, the future use of allo-MSCs has significant clinical potential. Hurdles exist, but many have already been overcome.

Cellular Cardiomyoplasty

Loss of cardiomyocytes after myocardial infarction (MI), combined with the absence of endogenous repair mechanisms, is a causative factor in progression to heart failure. Pathologic ventricular remodeling ensues as damaged myocardium is replaced by a fibrous scar composed of extracellular matrix produced by nonmyocytic cells, resulting in ventricular function loss.

In an effort to replace cardiomyocytes lost after ischemia, cellular transplantation has been investigated as a potential therapy for MI.[60] Termed cellular cardiomyoplasty, the approach has gone from an interesting research novelty to clinical reality. Adult cardiomyocytes have essentially no regenerative capacity, and although a cardiac stem cell has been described recently,[61] its physiologic role in repair after infarction appears minimal and functionally inadequate. Therefore, implantation of exogenous cells may allow for meaningful replacement of damaged cardiac cells. Various types of isolated cells have been delivered to the heart, including fetal cardiomyocytes,[62–64] skeletal myoblasts,[65–67] and more recently, progenitor or stem cells such as those of the endothelial[9] or mesenchymal lineages.[68–71] Studies evaluating these cell types have documented repeatedly that exogenous cells can engraft in adult myocardium and, in some cases, have a measurable functional impact on damaged myocardium. Additionally, these studies have addressed fundamental questions that must be considered when evaluating MSCs for cardiac cell therapy. For example, issues involving delivery strategies and the spatial placement of the cell graft, the ideal temporal relationships between cell delivery and cardiac injury, and the electromechanical properties of cell grafts have been addressed with fetal cardiomyocyte and skeletal myoblast grafts. Research with fetal cardiomyocyte grafts has been curtailed because of the improbable likelihood of procuring sufficient amounts of fetal cardiomyocytes thought to be necessary for the damaged adult heart.

In the early 1990s, Chiu et al[72] demonstrated the ability of skeletal myoblasts to be isolated, expanded in culture, and successfully delivered to injured myocardium. Since this pioneering work, skeletal myoblasts have been examined as a

cellular therapy in animal models of cardiac injury and are currently being tested in clinical trials in humans.[6] Skeletal myoblasts can be isolated from fetal, neonatal, and adult muscle and expanded in tissue culture.[73] However, myoblasts are committed progenitor cells and have been shown to differentiate to mature skeletal myocytes, not cardiomyocytes, when implanted into normal and injured myocardium.[60,63,65] That is, there is not current evidence of skeletal myocytes transdifferentiating to a cardiomyocyte phenotype after implantation in the heart.[65] Inherent differences in action potential generation and refractory period between these fiber types, alterations in cardiac conduction, and potential arrhythmogenesis may limit the long-term clinical utility of skeletal myoblasts.

MSCs for Cardiomyoplasty

Interest in MSCs, in general, and as a cardiac therapy, in particular, has increased exponentially during the past 5 years. Compared with other cells types considered for cardiomyoplasty, MSCs appear to possess unique properties that may allow for convenient and highly effective cell therapy. MSCs can be used allogeneically, delivered systemically, and differentiate into a cardiomyocyte-like phenotype when implanted in healthy myocardium.[28,69] Furthermore, MSCs can be readily transduced by a variety of vectors and maintain transgene expression after in vivo differentiation.[69,74] Transgene expression by MSCs may be used ultimately to augment cell engraftment or the extent of differentiation. Recent data from Mangi et al[75] support this possibility. This group used a retroviral vector to overexpress the prosurvival gene Akt in MSCs before implantation in infarcted rat myocardium. Akt protein overexpression was reported to greatly enhance MSC survival and prevent pathologic remodeling after infarction, with impressive improvement in cardiac output.

The first use of bone marrow cells for cardiomyoplasty was reported in 1999 by the laboratories of Weisel and Lee at the University of Toronto.[70] In this study, autologous bone marrow cells were implanted in the left ventricle (LV) of rats by direct injection 3 weeks after cryoinjury. Transplanted marrow cells could be identified in all animals 8 weeks after injury and were found to express muscle-specific proteins not present before implantation. Furthermore, improved systolic and diastolic functions were reported in animals that received cells pretreated with the DNA-demethylating agent 5-azacytidine, which has been reported to enhance myogenic differentiation of pluripotent cells. It should be noted that bone marrow, as opposed to a purified MSC population, was used in this study. Development of a causative relationship between the observed functional improvements and a particular cell population is problematic because of the fact that in addition to being a major repository for MSCs, whole marrow contains a number of cell types.

As opposed to the muscle precursor cells, allo-MSCs have the ability to be used immediately after acute injury.[76] The ability to treat MI patients with allo-MSCs in an emergent setting at the time of coronary reperfusion may constitute a distinct clinical advantage over autologous cellular cardiomyoplasty. Furthermore, MSCs appear to have the ability to home to the site of myocardial injury when administered intravenously after acute infarction (see below).

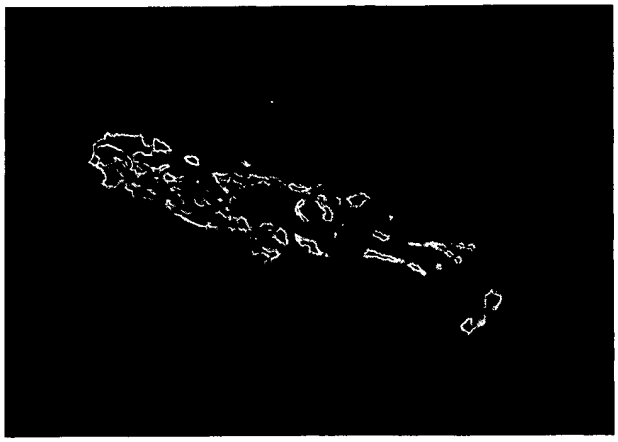

Figure 2. hMSC present in the ventricle wall of an adult mouse heart. The hMSC is identified with an antibody to transduced gene product β-gal (green), and the heart section has been counterstained with an antibody to desmin (red).

Pathologic Remodeling Inhibition After Infarction

Our laboratory and others have demonstrated that after injection into infarcted myocardium, engrafted MSCs differentiate toward a myogenic lineage, as evidenced by expression of muscle-specific proteins including α-actinin, troponin-T, tropomyosin, myosin heavy chain–MHC, phospholamban, and other muscle-specific proteins (Figure 2).[77] Additionally, the presence of connexin-43, a protein responsible for intracellular connection and electrical coupling between cells, further suggests cardiomyocyte differentiation. However, complete myogenic differentiation with mature sarcomeric organization, intercalated discs, etc, has not been observed after implantation of MSCs in infarcted myocardium. This is in contrast to our experience regarding hMSC injection into viable, adult, noninfarcted murine myocardium, where evidence of sarcomeric organization and extensive cardiomyogenic differentiation has been observed.[69] The lack of extensive myocytic differentiation in infarcted heart likely speaks to the importance of the local extracellular milieu in driving differentiation of MSCs.

However, even without appropriate differentiation within infarcted tissue, MSC cardiomyoplasty has been associated with a number of significant functional improvements in the postinfarcted heart. We described previously the benefits of engrafted MSCs including prevention of pathologic wall thinning (30% improvement in end-diastolic wall thickness at 12 weeks) and improved postinfarction hemodynamics (Figure 3).[77] Significantly lower end-diastolic pressure in MSC-treated animals (≈50% reduction in left ventricular end-diastolic pressure at 6 months) suggests improved diastolic relaxation and decreased wall stress that are likely attributable to favorable ventricular remodeling.[78] Absence of improvement in systolic function may be considered somewhat expected in light of the absence of sarcomeric organization in MSCs implanted in the infarct. Together, these data suggest that the implantation of MSCs in damaged myocardium

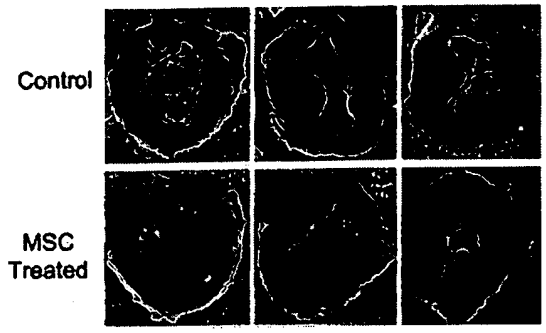

Figure 3. Unretouched photographs of the LV (cross-sections) at the level of MSC injection. These data provide evidence supporting the hypothesis that cellular cardiomyoplasty may limit LV wall thinning. Control hearts (top) had significant scarring, wall thinning, compensatory hypertrophy in adjacent myocardium, and loss of normal ventricle chamber geometry (dilation). In contrast, hearts of MSC-treated animals (bottom) had preserved wall thickness and LV geometry, despite equally large infarctions. Reprinted from Shake et al,[77] with permission from the *Society of Thoracic Surgeons.*

results in a more compliant, less stiff ventricle with improved diastolic-filling properties. How implantation of MSCs improves diastolic function mechanistically is not yet clear, but early evidence suggests that improved pathological remodeling, specifically alterations in tissue cellularity and extracellular matrix remodeling, may be involved. Although patients with isolated left ventricular diastolic dysfunction have lower mortality compared with those with systolic dysfunction, significant morbidities are common, including symptoms of recurrent chest pain and congestive failure, and diastolic benefits of MSC cardiomyoplasty may play an important role in ischemic heart disease treatment.

Allogeneic Use of MSCs in Cellular Cardiomyoplasty

As described previously, MSCs from human and other species have a cell surface phenotype that is of low immunogenicity.[21,50,79] In 2000, data from several research groups demonstrated long-term allo-MSC engraftment in a variety of noncardiac tissues in the absence of immunosuppression.[52,58,80] On the basis of these observations, our studies began to investigate the ability of allo-MSCs to engraft in infarcted myocardium. Initially in rats, and later in swine, allo-MSCs were found to readily engraft in necrotic myocardium and favorably alter ventricular function after infarction. Furthermore, allo-MSC engraftment in the myocardium occurs without evidence of immunologic rejection or lymphocytic infiltration, even in the absence of immunosuppressive therapy. These results using allo-MSCs further emphasize some of the apparent advantages of these cells over other cell populations for cellular cardiomyoplasty. These MSC characteristics may allow for enhanced clinical applicability because allo-MSCs can be readily available and administered with immunologic acceptance by the recipient.

The immunologically privileged status of MSCs may even extend to the xenogeneic setting. In a recent study from Saito et al,[81] MSCs obtained from C57BL/6 mice were injected intravenously into immunocompetent adult Lewis rats. Labeled mouse cells engrafted into the bone marrow for at least 12 weeks without immunosuppression. When these animals were later subjected to MIs, murine MSCs could be identified in the region of necrosis, and these cells expressed muscle-specific proteins not present before coronary ligation. Xenogeneic MSC engraftment was not associated with immunologic activation or rejection, but rather it appeared to form a stable cardiac chimera.

MSC Role in Angiogenesis

Because cardiac function depends critically on myocardial perfusion, restoration of cardiac function after MI must require not only replacement of lost cardiomyocytes but also revascularization of the injured region. Also, there is evidence from ischemic hind-limb models that circulating stem cells are incorporated into newly formed blood vessels and contribute to increased capillary density and enhanced tissue perfusion.[82,83]

Recent studies have also shown that stem and progenitor cells isolated from adult tissues have the potential for enhancing neovascularization and regenerating infarcted myocardium.[9,70,82,84–88] Myocardial perfusion is characterized by close matching of blood flow to myocardial demand. Therefore, it will be important to examine not only maximal blood flow (indicative of total vascularity) but also coronary flow responsiveness to changes in myocardial demand and the distribution of myocardial perfusion.

Experiments to evaluate the effect of MSC therapy on myocardial perfusion are ongoing. However, evidence for MSC involvement in vascular repair or regeneration can be found in Figure 4. The image was obtained from a pig 8 weeks after catheter-based endocardial delivery of allo-MSCs. The hematoxylin/eosin (H&E) images in the top panels clearly illustrate blood vessel presence within a region of generalized myocardial necrosis. In the confocal images of serial sections (bottom panels), 4′,6-diamidino-2-phenylindole (DAPI)-labeled cells can be seen throughout the section. However, localization of implanted MSCs can be readily identified surrounding and associated with these blood vessels. DAPI-labeled MSCs (blue) were localized within and intimately associated with the smooth muscle layer of the vessel. In addition to smooth muscle actin, implanted porcine MSCs have been demonstrated to express the von Willebrand factor, vascular endothelial growth factor (VEGF), and other proteins indicative of angiogenesis. These proteins are not found in cultured MSCs, but rather are expressed only after several weeks in the cardiac environment. Recent data from Kinnaird et al[88] suggest that the mechanism of MSC-mediated improvements in perfusion may reside in their ability to secrete a variety of angiogenic cytokines (fibroblast growth factor, VEGF, matrix metalloproteinases, platelet-derived growth factor, TGF-β, interleukin 1, angiopoetin, and others), many of which are upregulated with hypoxia.

MSC Cardiac Homing to Infarction Sites

One of the most interesting characteristics of MSCs is their ability to home to sites of tissue damage or inflammation.[89] Although the cytotactic factors responsible for injury-specific MSC migration and its physiologic consequences have yet to

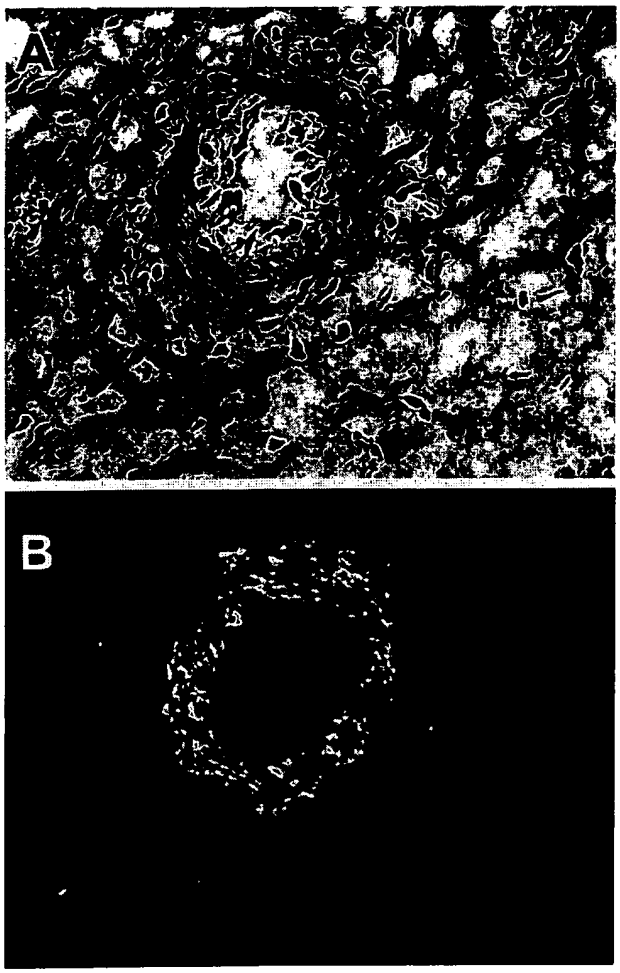

Figure 4. Immunohistochemical staining suggestive of MSC participation in postinfarction angiogenesis. A is an H&E-stained section of myocardium immediately adjacent to infarction (bottom) and illustrates a developing arteriole. A serial section viewed under confocal microscopy is shown in B. MSCs were labeled with DAPI before their intravenous injection 10 minutes after ischemia/reperfusion in a rat. DAPI-positive cells (blue) home to infarcted tissue and can be seen throughout the field, but only those integrated with the developing vessel are positive for smooth muscle actin (green).

be fully elucidated, it is postulated that MSCs migrate to participate in wound repair. This extraordinary ability of MSCs to home to sites of acute tissue injury has been demonstrated in the settings of bone fracture[74,89,90] and cerebral ischemia,[91] as well as the infarcted heart.[81,92]

Saito et al[81] were the first to demonstrate that MSCs administered intravenously engraft within regions of MI. In this study, mouse MSCs were transduced with a LacZ reporter gene and injected intravenously to rats (in the absence of immunosuppression). In healthy (noninjured) animals, intravenous MSCs preferentially engraft in the marrow cavity. However, when these rats were subjected to a cycle of ischemia/reperfusion, significant numbers of labeled xenogeneic MSCs could be identified in the circulation and, subsequently, the infarcted region of the heart. The majority of engrafting MSCs were found to be positive for cardiomyocyte-specific proteins, whereas a distinct subpopulation was determined to participate in angiogenesis.

Our laboratory has used a similar model in an attempt to characterize MSC movements after intravenous administration. MSCs obtained from the ACI rat strain were transduced with a retroviral vector encoding for β-galactosidase (β-gal) expression. These labeled MSCs were identified within the bone marrow of Fisher rats 10 days after intravenous injection, and MSCs were not detected in the heart or lungs at that time. When rats were submitted subsequently to a 45-minute cycle of left anterior descending artery occlusion and reperfusion, a significant number of β-gal–positive MSCs were detected within the infarction region (Figure 5A). This engraftment was highly specific in that labeled MSCs were not detected in noncardiac tissues or the viable myocardium of the right ventricle, atria, or LV posterior wall.[93]

Although these data shed some light on the physiology of MSCs in wound repair, the question remained whether MSCs administered intravenously after infarction could similarly home to and possibly aid in repair of damaged myocardium. When administered during the acute reperfusion period, this in fact appears to be the case.[93,94] In a rat infarct model, administration of 5 million MSCs intravenously after 10 minutes of reperfusion resulted in significant and sustained cardiac engraftment. However, the "window" of MSC homing is limited. If MSC administration was delayed until 2 weeks after infarction, no significant cardiac engraftment was observed, with most cells returning to the bone marrow (Figure 5B). The remarkable specificity with which MSCs home to regions of injury after systemic delivery with essentially no engraftment in normal tissue is illustrated in Figure 6. Although the factors responsible for MSC migration have not yet been defined clearly, the transient nature of the phenomenon is consistent with the involvement of an inflammatory mediator similar to those responsible for macrophage and neutrophil infiltration in injured tissue. Further complexity is added by a recent report suggesting that expansion of murine MSCs in culture may diminish the efficacy of injury-induced homing.[95] The degree to which species differences may account for these varying results requires further investigation.

Tracking and Quantification of Engrafted MSCs

One of the problems inherent to the study of cellular cardiomyoplasty, particularly with intravenous administration, is the difficulty in tracking the movement of implanted cells. Recently, Barbash et al[96] used γ camera imaging of ^{99m}Tc-labeled MSCs to determine their distribution after intravenous or intraventricular injection in a rat MI model. They reported that 4 hours after intravenous injection, a significant number of MSCs were trapped in the lungs, with lesser numbers in the heart and other organs. Cardiac engraftment was augmented significantly (and pulmonary plugging attenuated) with intraventricular administration. MRI of iron-labeled cells is another technique that has been used recently to track MSC distribution in vivo.[97,98] Hill et al[98] demonstrated that iron labeling does not alter MSC multipotentiality and that imaging in a standard 1.5-T magnet provides excellent special resolution in the beating heart. Furthermore,

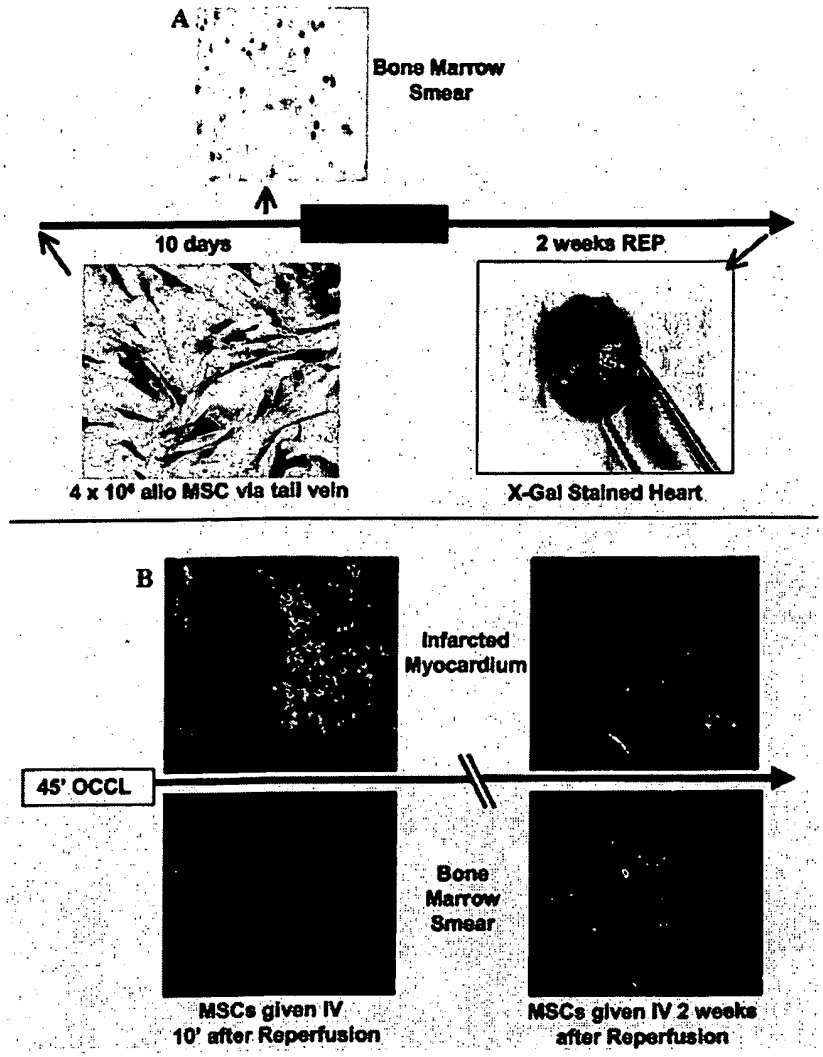

Figure 5. A, Rat MSCs were transduced in vitro with the β-gal reporter gene to follow their distribution after intravenous delivery. Left, Nearly 100% of the transduced MSCs stained positive for β-gal activity before injection; 3.9×10^6 MSCs were injected into the tail vein of healthy Fisher rats. β-Gal–positive cells were only detected in the bone marrow 10 days after MSC delivery. When these animals were subjected to 45 minutes of ischemia and 2 weeks of reperfusion, labeled MSCs migrated to the site of infarction. Labeled cells were not detected in noncardiac tissues at 2 weeks after infarction. B, To determine whether MSCs could home to sites of infarction when administered systemically at reperfusion, 2×10^6 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI)-labeled rat MSCs were delivered intravenously either 10 minutes (left) or 14 days (right) after reperfusion. In both groups, animals were euthanized 14 days after cell injection, and the hearts (top) and bone marrow (bottom) were examined for the presence of DiI-positive MSCs. Qualitative assessment suggests that MSC delivery at 10 minutes after reperfusion results in a greater degree of MSC engraftment in the infarcted myocardium than delivery at 14 days. Conversely, MSC engraftment in the bone marrow is increased with delayed administration.

the iron label can be conjugated to a fluorescent particle to aid in histologic MSC identification.

MSC localization in vivo with either MRI or γ imaging is of tremendous value to those attempting to demonstrate the biodistribution or persistence of MSC cardiomyoplasty. However, the inability of the techniques to accurately quantify the number of MSCs is troubling to those seeking to optimize the therapeutic use of MSCs. When attempting to calculate dose/response relationships in such studies, it is the number of cells engrafting that is of concern, as opposed to the number administered. Accurate cell engraftment quantification has been elusive regardless of cell type examined. Some investigators have attempted to generate indexes of engraftment by simply counting the number of labeled cells in a representative sampling of high-powered fields. This methodology is hindered by the possibility of counting multiple cell layers or fractions of cells and, therefore, is not exact. We have attempted to quantify MSC engraftment using RT-PCR amplification of transgene expression. Although theoretically sound, in our experience, PCR-based quantification of trans-

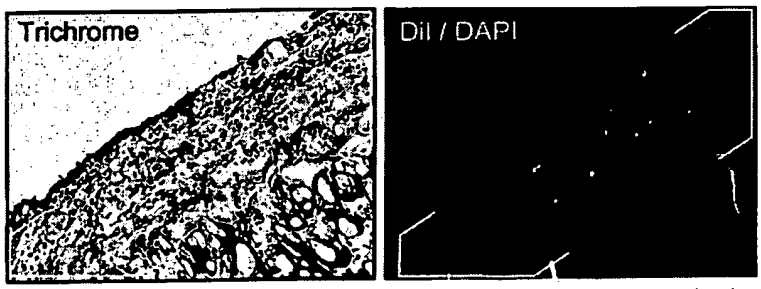

Figure 6. Histologic analysis was used to determine the specificity with which MSCs home to regions of infarction after intravenous delivery. Rat MSCs were labeled with DAPI (blue fluorescence) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI; red fluorescence) in culture immediately before injection. Left, Infarcted tissue is identified in trichrome-stained samples by blue-green coloration (yellow brackets). Note region of viable myocardium in bottom right portion of image. Right, Fluorescently labeled MSCs were clearly identified in serial tissue sections using confocal microscopy. Comparison of fluorescent images to trichrome-stained sections demonstrated a high degree of correlation between the region of MSC engraftment and the region of necrosis. Essentially, no cells were identified in regions of viable myocardium or noncardiac tissues.

duced MSCs lacks the sensitivity required for accurate tissue quantification when the number of engrafted cells is low.

A method found to provide a more accurate quantification of MSC engraftment is radiolabeling of MSCs with tritiated thymidine ([^{3}H]-thymidine). After implantation, the degree of radioactivity in a tissue can be correlated to MSC engraftment in a linear fashion. Although PCR techniques would allow for detection of 50 000 cells in a rat heart, tritium labeling has a 10-fold lower threshold of detection (≈5000 cells), greatly improving accuracy of measurements as well as our ability to detect modest numbers of cells in the heart.

Using these types of techniques has allowed investigators to quantify the degree of MSC engraftment in the heart after delivery. Although the exact numbers vary, it can be said that in all cases, the extent of engraftment is currently modest. Some experiments have indicated that <3% of MSCs administered by direct injection persist after 2 weeks. Administration of higher numbers of cells results in only modest augmentation of long-term engraftment.

Summary

Adult stem cells are found in many tissues and participate in adult growth as well as damaged tissue repair and regeneration. The cellular and tissue environment in the adult is likely very different from the early embryo conditions that produce ES cells. Bone marrow provides an accessible and renewable source of adult MSCs that can be expanded greatly in culture and characterized. Culture-expanded and characterized MSCs have been tested for their ability to differentiate into several lineages in vitro and also tested in animal models for their ability to enhance tissue repair and undergo in vivo differentiation. One of the greatest attributes of MSCs is their potential to supply growth factors and cytokines to repairing tissue. MSCs do not appear to be rejected by the immune system, allowing for large-scale production, appropriate characterization and testing, and the subsequent ready availability of allogeneic tissue repair–enhancing cellular therapeutics. This provides for the further development of this new field and paves the way for the use of yet other stem cells. The potential to use MSCs to repair damaged cardiovascular tissue is very promising and moving forward quickly. The current results from many labs and early cardiac clinical studies suggest important therapeutic approaches will be forthcoming through MSC use. Perhaps most important, understanding adult stem cells such as MSCs will provide us with greater insight into human biology.

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